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Long-term conservation of *Diospyros* germplasm using dormant buds by a prefreezing method

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Abstract

Cryopreservation of *Diospyros* at -150℃ followed by a prefreezing treatment using dormant winter buds was investigated. Twig sections bearing one bud each were first partly dehydrated at room temperature for 3 h. Then they were subjected to a successive prefreezing at -5, -10, -15, -20 and -30℃ at 24 h intervals, and finally preserved at -150℃ in a deep freezer. Thawing in the air at 25℃ gave the highest rate (about 70%) of *in vitro* shoot formation from cryopreserved buds. Shoot regeneration by bud grafting was not successful. Dormant buds appear to be a suitable material for long-term storage of some *Diospyros* species.

Key words: cryopreservation, dormant bud, *Diospyros*, persimmon, prefreezing method

Diospyros contains about 400 species and is distributed in the tropics of Asia, Africa and Central-South America. Only a few species native to the temperate zone have been cultivated, the most common persimmon (*Diospyros kaki* Thunb.). A group of outlying warm north temperate species (*D. kaki*) is widely cultivated in Japan, China and Korea, and is also found in west Asia. *D. lotus* and *D. virginiana* occur in North America (Yonemori *et al.*, 2000). All are conserved entirely in field genebanks in some repositories. Cryopreservation is recognized as having the distinctive advantage of allowing long-term conservation with minimum space and maintenance requirement (Sakai, 1997). There is only one report of successful cryopreservation of *Diospyros* germplasm accomplished by vitrification (Matsumoto *et al.*, 2001). In this report, we describe a simpler and reliable protocol for cryopreservation of *Diospyros* germplasm using dormant buds by a prefreezing method.

Dormant axillary vegetative buds of persimmon (*Diospyros kaki* Thunb. cv. 'Saijo') were used in this study unless stated otherwise. Twigs with dormant vegetative buds were collected from a 20-year-old tree in the orchard of Shimane Agricultural Experiment Station in early of January when the buds were still in a state of quiescence under daily mean temperatures at 10 to -5℃. After storage at 0℃ for 1 month, they were cut into 3-cm pieces that contained one bud each and partly dehydrated at 25℃ for 3 h (the fresh weight decreased about 3%).

Ten twigs were placed in a 50 ml polypropylene tube, and they were first cooled at -1℃/min to -5℃ and kept at this temperature for 1 day using a programmable freezer with methyl alcohol. They were then successively cooled at the same rate to -10, -15, -20 and -30℃ and kept at each temperature for 1 day in the freezer. The tubes were finally transferred into a deep freezer at -150℃ and held there for at least 5 days. To determine the optimal thawing temperature, cryopreserved twigs in a 50 ml polypropylene tube were thawed at -1℃, at 25℃ in an air conditioned room for 24 h or at 40℃ in a water bath for 15 min and then held at room temperature (25℃) for 24 h. Thawed twigs were sterilized in 70% ethanol for 2 min and in 2% sodium hypochlorite solution containing 0.01% Tween 20 for 20 min and rinsed three times in sterile distilled water. We previously showed that about 95% of winter buds of 'Saijo' can survive after this sterilization treatment using buds stored at

-196℃ by vitrification method (Matsumoto *et al.*, 2001). It appeared that the winter buds of 'Saijo' are covered with scales and several young leaves, and thus can tolerate this sterilization procedure.

Shoot tip sections of approximately 1 mm long were excised from the sterilized twigs and plated on a modified Murashige and Skoog (1962) basal medium (half strength of KNO_3 and NH_4NO_3, termed 1/2 MS medium) containing 1 mg l^{-1} zeatin, 3% sucrose and 0.2% gellan gum at pH 5.8 in Petri dishes (9 cm in diameter). They were cultured under white fluorescent light (50 μ mol s^{-1} m^{-2}), 16 h photoperiod at 25℃. The rates of shoot formation and survival of cryopreserved and thawed winter buds were determined 30 days after plating.

As shown in **Fig. 1**, shoot tips derived from dormant buds thawed at 25℃ gave the highest rate of shoot formation (70%) and the lowest rate of death (30%). The rate of shoot formation was about 40% for samples rapidly thawed at 40℃ in a water bath, a method commonly used for vitrification and encapsulation-dehydration. Slow thawing at -1℃ in the air resulted in the lowest rate of shoot formation (28%) and the worst rate of death (50%). Thus cryopreserved dormant buds of persimmon are injured either by too rapid or too slow thawing.

Although all of the cryopreserved and thawed shoot tips turned brown after plating, many of them resumed growth within 20 days and developed from the shoot tip region within 50 days (**Fig. 2**). The prefreezing-treatment-based cryopresevation protocol established here was applied to four other

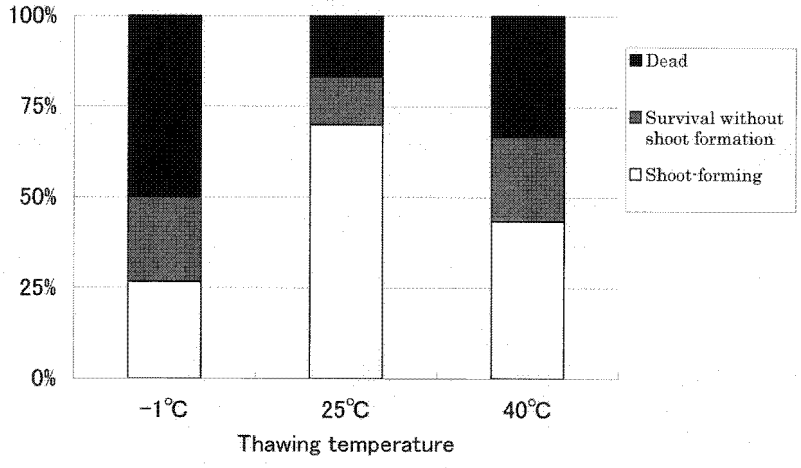

Fig. 1 Effect of thawing temperature on shoot formation and survival of shoot tips excised from dormant buds of *Diospyros* after cryopreservation by a prefreezing method.
Cryopreserved dormant buds of Japanese persimmon cv. 'Saijo' were thawed at -1℃, 25℃ in an air conditioned room for 24 h or at 40℃ in a water bath for 15 min following holding at room temperature (25℃) for 24 h. After sterilization, shoot tips were removed from the dormant buds and plated on solidified 1/2 MS medium containing 1 mg l^{-1} zeatin. The rates of shoot formation and survival of cryopreserved and thawed shoot tips were determined 30 days after plating. Approximately 10 shoot tips were tested for each of three replicates..

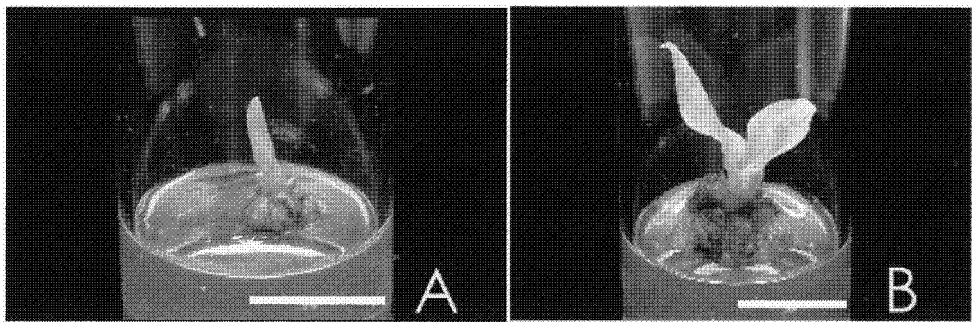

Fig. 2 Development of *D. kaki* (cv. 'Saijo') dormant shoot tips after cryopreservation by a prefreezing method.
Cryopreserved twigs were thawed in the air at 25℃ for 24 h and derived shoot tips after sterilization. Shoot tips after 20 days (A) and 50 days (B) of reculture. Bar = 10 mm.

Table 1 Percentages of survival and shoot formation of dormant shoot tips from temperate and subtropical cultivars or species of *Diospyros* cryopreserved by a prefreezing method.

Cultivars - Species	*Survival	Shoot formation (% ± SE)
Temperate *D.*		
D. kaki Thunb.		
Mopan	50.0 ± 5.8	3.3 ± 3.3
Zhugguodenglonghong	53.3 ± 3.3	0
Gongchengshuishi	30.0 ± 5.8	56.7 ± 6.7
Qujingshuishi	48.1 ± 1.9	24.8 ± 6.8
Subtropical *D.*		
D. taitoensis Odashima	36.7 ± 3.3	0
D. morrisiana Hance	56.5 ± 9.4	0

*Survival: Survival without shoot formation. Approximately 10 shoot tips were tested for each of three replicates

cultivars of temperate *Diospyros* ('Mopan', 'Zhugguodenglonghong', 'Gongchengshuishi' and 'Qujingshuishi') and to two subtropical *Diospyros* species (*D. taitoensis* and *D. morrisiana*). The rate of shoot formation from cryopreserved buds varied markedly among the *Diospyros* cultivars and species tested (**Table 1**). In the two subtropical *Diospyros* species and 'Zhugguodenglonghong' cultivar, no shoot formation occurred but the rate of survival without shoot formation was 36.7 - 56.5%. This suggests that portions of the meristimatic dome were injured during the cooling or thawing processes in them. Harada (1985) reported that dormant buds of Japanese persimmon cv. 'Hiratanenashi' survived at -15℃. In our present study with 'Saijo', dormant buds survived at -30℃ during the prefreezing process. These results suggest that the key of successful cryopreservation for *Diospyros* by the prefreezing method depends on the cold tolerance of *Diospyros* species or cultivars at -30℃.

For successful cryopreservation, it is essential to avoid lethal intracellular freezing, which might occur when plant cells were directly plunged into liquid nitrogen (Sakai and Yoshida, 1967). Thus, a sufficient dehydration of cells and shoot tips, which is done by vitrification or encapsulation-dehydration method, prior to the transfer to liquid nitrogen has to be made to minimize or avoid intracellular freezing in any cryogenic procedures. However, dehydration may accompany harmful effects due to osmotic stress and chemical toxicity. In order to increase the dehydration tolerance, shoot tips are therefore subjected, before dehydration treatment, to cryoprotective treatments such as preconditioning (ex. cold hardening), preculture with 0.3 M sucrose for 1-3 days and treatment with LS medium supplemented with 2 M glycerol and 0.4 M sucrose (Sakai, 2000). It should be noted however that these cryoprotective treatments make cryogenic procedures more complex, and that a simpler cryogenic procedure is desirable for plant germplasm conservation.

There are several reports on cryopreservation using dormant winter buds of apple (Tyler and Stushnoff, 1988a, b; Forsline *et al.*, 1998), blueberry and raspberry (Niino *et al.*, 1990), mulberry (Yakuwa and Oka, 1988; Niino *et al.*, 1993), pear (Suzuki *et al.*, 1997), cherry (Towill and Forsline, 1999) and *Diospyros* (Matsumoto *et al.*, 2001). Dormant buds have high level of tolerance to dehydration and cold temperature stress, and, thus, are very suitable materials for cryopreservation. Niino *et al.* (1993) reported that one of the advantages of dormant winter buds as the material for cryopreservation is that it is tolerant to toxic effects of chemical cyoprotectants. Cryopreservation using dormant buds of several *Malus* species is being utilized at USDA-ARS National Center for Genetic Resources Preservation in Fort Collins (Towill *et al.*, 2002).

Niino *et al.* (2000) and Towill *et al.* (2002) regenerated shoots from cryopreserved dormant buds of mulberry and apple by bud grafting. However, in our present study, no shoots were formed from grafted buds of cryopreserved twigs. Thus, further study is necessary in the prefreezing-method-based cryopreservation of *Diospyros* dormant winter buds.

In conclusion, the prefreezing method using dormant buds presented in this study seems to be a simple, reliable and practical protocol for cryopreservation of *Diospyros* germplasm. It requires neither *in vitro* materials and nor special skills in the cryogenic procedure. Dormant buds may promise to be a very practical system for long-term storage of

many kinds of temperate woody plants.

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